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Immunogenicity of Peptide-vaccine Candidates Predicted by Molecular Dynamics Simulations

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We present an *in silico*, structure-based approach for design and evaluation of conformationally restricted peptide-vaccines. In particular, we designed four cyclic peptides of ten or 11 residues mimicking the crystallographically observed β-turn conformation of a predicted immunodominant loop of PorA from *Neisseria meningitidis*. Conformational correctness and stability of the peptide designs, as evaluated by molecular dynamics simulations, correctly predicted the immunogenicity of the peptides. We observed a peptide-induced functional antibody response that, remarkably, exceeded the response induced by the native protein in outer membrane vesicles, without losing specificity for related strains. The presented approach offers tools for *a priori* design and selection of peptide-vaccine candidates with full biological activity. This approach could be widely applicable: to outer membrane proteins of Gram-negative bacteria, and to other epitopes in a large range of pathogens.

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Keywords: peptide-vaccine; molecular dynamics; structure-based design; immunogenicity; beta-turn

Peptides may have potential to afford safe, non-infective, well-defined and stable vaccines. However, induction of functional humoral immunity with unstructured peptide vaccines may be difficult. Even if the protein epitope comprises a continuous amino acid sequence, peptides containing this sequence may induce antibodies that lack cross-reactivity with the parent protein or whole microorganism. Molecular mimicry to the cognate antigen seems to be required for inducing a functional, cross-reactive immune response.[1] In several studies this functional molecular mimicry has been achieved by a trial-and-error approach,[2–7] by combinatorial chemistry display techniques[8,9] or by structure-based design.[10–12] Similar to approaches in structure-based drug design, crystal or solution structures of peptide-antibody complexes are often used to optimize the complementarity between a peptide and a functional antibody, which should thus lead to higher binding affinity.[13] However, it is not the binding to an antibody that is important for the design of immunogenic peptides, but rather the molecular mimicry of the unbound peptide to the cognate antigen.[14] This cannot be optimized by modeling a peptide into an antibody structure, but rather by investigating the conformational properties of the peptide.[10,11] Irrespective of the method used for obtaining the designs, peptide-vaccine candidates have to be tested to validate their competence *in vivo*.[14] However, prediction of the conformational
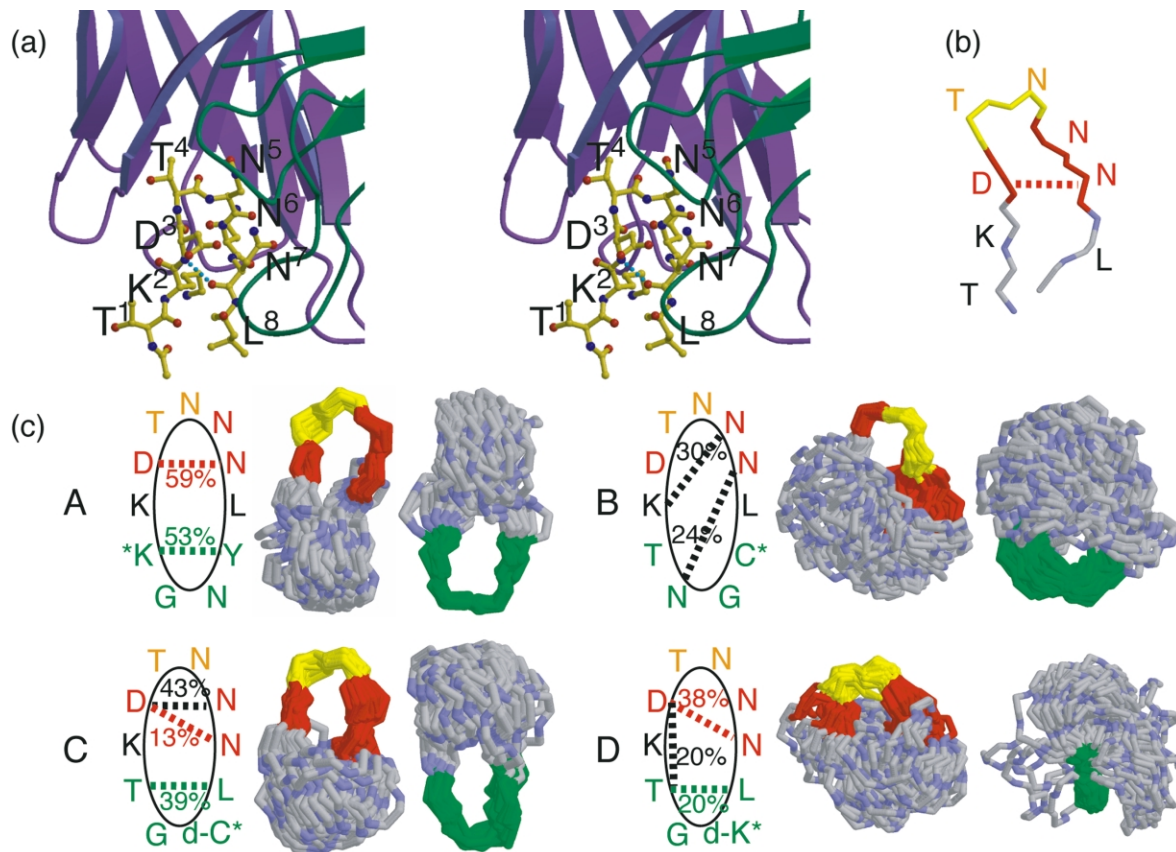
Figure 1. Target conformation, *in silico* evaluation of peptide designs. (a) Stereo view and (b) backbone conformation of the linear epitope peptide showing a 3:5 type I β-turn at residues ^{P3}DTNNN^{P7} (corresponding to residues 182–186 in PorA), as observed in complex with a Fab fragment from a bactericidal antibody against *N. meningitidis* subtype P1.16 in a crystal structure.[17] Peptide-vaccine candidates were designed to mimic this conformation; designs are represented schematically using the one-letter code for amino acids in (c); asterisks indicate the site for attachment to carrier protein tetanus toxoid. To reduce the flexibility, the peptides were conformationally restricted by head-to-tail cyclization. The residues of the contra-turn were chosen to have a high propensity to form a β-turn, thus stabilizing the epitope-turn conformation by prolonging the hydrogen-bonding network. Molecular dynamics simulations of the peptides were done to assess the conformational mimicry of the peptides to the crystallographically observed β-turn. (c) Snapshots of the peptides during the molecular dynamics simulation run were superimposed on either epitope-turn (red and yellow) or contra-turn (green). The conformational mimicry is assessed by the presence in the simulation of the desired backbone (red and green dotted lines) and undesired (black dotted lines) hydrogen bonds; the percentages of simulation time for observed hydrogen bond formation are included in the representation of the designs. For the molecular dynamics simulations, the molecular models of the peptide designs were constructed with the desired contra-turn and energy minimized using InsightII (version 98, Accelrys Inc.). The conformational properties of the cyclic peptides were investigated by molecular dynamics using the GROMOS96 package.[31] The simulations were run under NPT conditions in explicit solvent for 10 ns at 400 K as described.[32] Simulations were analyzed by GROMOS96 analyzing programs, using common hydrogen-bond restrictions (donor–acceptor distance ≤2.5 Å, donor–hydrogen–acceptor angle ≥135°).

properties of the peptides could *a priori* select the most promising peptide designs for further *in vivo* testing.

Outer-membrane proteins display β-barrel structures with generally extended loops at the bacterial surface. These surface-exposed loops may be immunogenic and are often observed to form β-turn structures.[15] One example in *Neisseria meningitidis* is the abundant outer-membrane protein porin PorA, which is predicted to contain eight surface-exposed loops.[16] Most variability between PorA protein of different subtypes resides in three variable regions, VR1, VR2 and, less pronounced, VR3, which correspond to the predicted

loops 1, 4 and 5, respectively.[16] Monoclonal antibodies reacting with peptide epitopes located on loops 1, 4 (and 5) have been found to be bactericidal. This makes these epitopes good candidates for the development of subtype-specific peptide vaccines.

The PorA P1.16 subtype investigated in this study is a common subtype, which is located on loop 4. For this subtype, immunogenic, cyclic peptides of 15–35 amino acid residues have been discovered by empirical research.[5,6] The crystal structure of a Fab fragment from a bactericidal antibody specific for this subtype in complex with a linear peptide (^{P1}TKDTNNNL^{P8}, corresponding

to residues 180–187 in PorA)[17] has given opportunities for structure-based improvement of the peptide designs. Computational lead-optimization of the peptide using LUDI, however, did not yield peptides with higher binding affinities,[13] nor were these peptides tested for their immunogenicity. Though our approach is based on the same crystal structure, it does not optimize binding, but rather optimizes the molecular mimicry of the hairpin with a 3:5 type I β-turn at residues DTNNN (*a:b* nomenclature as used by Sibanda *et al.*[18] (Figure 1(a)). The peptide designs were conformationally restricted by head-to-tail cyclization and evaluated *in silico* by molecular dynamics simulations and *in vivo* by an immunization experiment in mice. Additionally, the affinities of the peptides for two bactericidal antibodies were measured.

Peptide design

We chose head-to-tail cyclization of the peptides to stabilize the crystallographically observed β-hairpin conformation.[17] Residues for cyclization were selected to have a high propensity to form a second β-turn, the contra-turn, prolonging the hydrogen bonding network[19−22] and hence stabilizing the β-turn of the epitope. A reverse turn conformation was chosen, because it provides the appropriate geometry, topological twist and in-register peptide alignment.[23] In addition, a high immune response requires conjugation to a carrier protein, for which we used tetanus toxoid. Thus, a site for conjugation was incorporated in the contra-turn either by a cysteine or by an N^ε-(S-acetyl-mercaptoacetyl)lysine residue (Lys-SAMA), both of which can be attached to a sulfhydryl-reactive protein.

Peptides A and B consisted of 11 residues (Figure 1(c)). The contra-turn of peptide A was designed to form a type I′ β-turn using the most commonly observed residues for this turn,[19,24] i.e. Tyr-Asn-Gly-Lys for position *i* to *i* + 3. The turn-stabilizing properties of asparagine and glycine are supported by experimental studies and molecular dynamics unfolding studies.[19,24−27] Unlike the contra-turn of peptide A, the contra-turn of peptide B was designed to form a type II′ turn with residues Cys-Gly-Asn-Tyr. The type II′ turn conformation is stabilized by the asparagine residue at position *i* + 2, which has a preference for adopting the ϕ,ψ conformation characteristic for this turn (ϕ = 80°, ψ = 0°).[19] Though a tyrosine residue at position *i* is preferred in this turn, a cysteine residue was chosen to introduce a site for conjugation.

Peptides C and D consisted of only ten residues (Figure 1(c)). D-Cysteine and D-lysine-SAMA were chosen for position *i* + 1 of peptides C and D, because D-amino acids are known to stabilize a reverse turn by favoring positive ϕ angles.[28] A glycine residue was chosen for position *i* + 2, as this amino acid can adopt the correct ϕ,ψ-angles (90°,

0° for type I′ and −80°, 0° for type II′). In order to have a higher overlap in sequences of peptide and protein, the amino acid residues at positions *i* and *i* + 3 were chosen to be the same as in the cognate protein, i.e. leucine and threonine respectively. These amino acid residues form a common pair in anti-parallel β-sheets[20] and are not uncommon in reverse turns.[19]

Assessment of peptide designs by molecular dynamics simulation

Molecular dynamics simulations of the four designed P1.16 peptides were performed to assess the conformational stability of the desired β-turn *in silico* (Figure 1(c)). In these simulations, the presence of the hydrogen bond between main-chain Asp[P3] NH and Asn[P7] CO was used as a criterion to assess the stability of the 3:5 type I β-turn. In peptide A, the designed 3:5 type I β-turn epitope conformation was observed for 58% of the time. Concomitantly, its contra-turn yielded a relatively stable type I′ β-turn present for 53% of the simulation time. These data were supported by solution NMR experiments, showing that the peptide was very flexible (chemical shifts close to random coil, no long-range nuclear Overhauser effects (NOEs)), but with medium-range NOEs that were in agreement with the presence of the epitope- and contra-turn (data not shown). In contrast, the epitope-turn conformation in peptide B was shifted by one residue, yielding a 2:4 turn for KDTN present 30% of the time. Overall, peptide B was highly flexible and no stable conformation was observed for the contra-turn. In peptide C, the desired 3:5 DTNNN turn conformation was observed for only 13% of the time. Instead, the dominant conformation of the epitope-turn was a 2:4 DTNN turn present for 43% of the time; the contra-turn was found to be relatively stable in a type I′ β 2:4 conformation present 39% of the time. Finally, peptide D was highly flexible with no stable contra-turn conformation, but still displayed a correct 3:5 DTNNN turn for 38% of the simulation time. In summary, the simulations indicated that: (1) peptide A displayed the correct conformation for 58% of the simulation and was the least-flexible peptide design; (2) peptide D displayed the correct conformation of the epitope turn for 38% of the simulation, but remained very flexible; (3) peptides B and C were predominantly in an incorrect conformation for the epitope. Therefore, *in silico* evaluation of the peptide designs put peptide A forward as the best potential vaccine candidate followed by peptides D, C and B.

Evaluation of peptide design by SPR-binding studies

An SPR-binding assay was used to determine the affinity of the designed peptides for two bactericidal antibodies. We observed large differences in binding affinity for the four peptides, in the
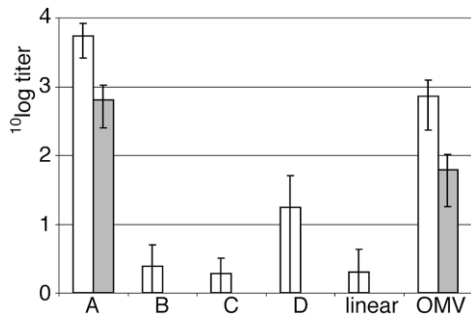
Figure 2. *In vitro* and *in vivo* evaluation of the peptide designs A to D and a linear control peptide. Binding to bacteria in whole-cell ELISA (open bars) and complement activation in a bactericidal assay (filled bars) of sera from immunization experiments in mice of the peptides conjugated to tetanus toxoid, and outer membrane vesicles (OMV), used as a positive control. Bactericidal antibodies were induced only by peptide-A conjugate and OMV, with highest titers for peptide-A conjugate, (both exceed the antibody response observed after natural infection). The immunization experiments, whole-cell ELISA experiments and serum bactericidal assays were performed as described,[6] using groups of minimally five BALB/c mice (20–24 g) per peptide conjugate. Unless stated otherwise, the vaccine contained 50 μg of peptide conjugate and 20 μg of Quillaja saponin A (Quil A) per mouse. Peptides were synthesized and purified as described,[6] and analyzed by HPLC/ion spray ionization mass spectrometry. For immunization, the peptides were coupled to bromoacetylated tetanus toxoid to give thioether conjugates, as described.[6] Additional binding experiments of the cyclic peptides A–D and the linear control peptide (Ac-YTKDTNNNLTL-NH$_2$) to a bactericidal antibody were performed using surface plasmon resonance. The BIAcore 2000 apparatus, CM5 sensor chip, HBS-EP buffer and amino coupling kit were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). The anti-P1.16 antibodies MN12H2[17] and MN5C11G[33] were coupled to the chip by standard amine coupling according to the manufacturer's instructions.[34] Different concentrations of peptides, ranging from 16 nM to 100 μM in HBS-EP buffer, were flowed across the surface at a rate of 5 μl/minute. Results were analyzed by BIAevaluation software 3.0 using a standard 1:1 Langmuir binding model, where appropriate with drifting baseline, and yielded K_D values for MN5C11G of: A, 16 nM ($\chi^2 = 0.63$); B, 32 nM ($\chi^2 = 0.62$); C, 500 μM ($\chi^2 = 0.36$); D 110 μM ($\chi^2 = 0.61$); and linear 57 nM ($\chi^2 = 0.56$); and K_D values for MN12H2 of: A, 19 nM ($\chi^2 = 0.48$); B 0.59 μM ($\chi^2 = 0.29$); C, 7.3 μM ($\chi^2 = 0.18$); D, 5.1 μM ($\chi^2 = 0.11$); and linear 0.96 μM ($\chi^2 = 0.11$). NMR experiments included standard nuclear Overhauser effect spectroscopy (mixing time 400 ms) and total correlated spectroscopy experiments (mixing time 80 ms) at 278 K using a sample containing approximately 0.2 mM peptide A in a 20 mM sodium phosphate buffer at pH 5.5 and 10% ^{2}H$_2$O on a Bruker AMX 600 MHz spectrometer.

Figure 3. Immunological properties of peptide-A conjugate: (a) Binding to bacteria (open bars) and bactericidal activity (filled bars) of sera induced by different doses of peptide-A conjugate, ranging from 2 μg to 50 μg, and by 1/10 human dose of OMV. Comparable titers were elicited by all doses of peptide conjugate ($p = 0.37$) and all peptide-induced titers were higher than the titers elicited by outer membrane vesicles (OMV) ($p < 0.01$). (b) Titers induced by the lowest dose of peptide-A conjugate (open bars) and OMV (filled bars) were analyzed for their cross-reactivity in whole-cell ELISA using different strains. The strains were obtained from the Netherlands Reference Laboratory for Bacterial Meningitis (AMC/RIVM) and from the RIVM collection; the sequence of loop 4 (VR2), the loop 1 (VR1) and the reactivity with monoclonal anti-P1.16 antibody MN5C11G are shown in Table 1. Subtypes are named as in the *N. meningitidis* PorA Variable Region Database. Based on the recognition by the peptide- and OMV-induced sera, strains were categorized in: (I) strains fully recognized by either of the sera: full cross-reactivity concerning the P1.16 epitope, (II) strains lowly recognized by OMV sera, but not by peptide sera; the cross-reactivity of the OMV-induced sera most likely comes from the P1.7 epitope as present in the P1.7,16 OMV, (III) strains which were not recognized by either of the sera: no cross-reactivity of the sera with the P1.16 variants.

and 0.6 when fit with a standard 1:1 Langmuir model).

Immunization trials

Immunization experiments in mice were performed for the four cyclic peptides A–D and a linear control peptide with sequence Ac-KDTNN NLYNG-(Lys-SAMA)-NH$_2$ conjugated to tetanus toxoid. As a positive control outer membrane vesicles (OMV), consisting for 90% of PorA

order of decreasing affinity A > B > linear peptide $\gg$ D > C for both antibodies (with K_D values for antibody MN5C11G of 16 nM, 32 nM, 57 nM, 110 μM and 500 μM, respectively, and of 19 nM, 0.59 μM, 1.0 μM, 5.1 μM and 7.3 μM, respectively, for antibody MN12H2 with χ^2 values between 0.1

Table 1. Strains and loop 4 sequences used in cross-reactivity experiments

Strain	Sero-group	Sero-type	Loop 1 sequence subtype[a]	Loop 4 sequence subtype[a]	Sequence loop 4[b]	Reaction with Mab anti-P1.16[c]	Category[d]
H44/76	B	15	P1.7	P1.16	YYTKDTNNNLTLVP	+	I
2000119	B	15	P1.7	P1.16-33	YY**T**NTKDTNNNLTLVP	+	I
2000644	B	4	P1.31	P1.16-34	YYTKDTNNNL**KDTNNNL**TLVP	+	I
MC58	B	15	P1.7	P1.16-2	YYTK**N**TNNNLTLVP	+/−	I
00260	B	15	P1.7	P1.16-6	YYTK**H**TNNNLTLVP	−	II
2000024	B	15	P1.7	P1.16-32	YYTK**V**TNNNLTLVP	−	II
2001411	B	15	P1.7	P1.16-35	YYTK**HA**NNNLTLVP	−	II
10402	C	15	P1.7	P1.16-29	YYTK**G**TNN**D**LTLVP	−/+	II
10904	B	Nt	P1.17	P1.16-4	YYTKD**KN**D**K**LTLVP	−	III
00298	B	Nt	P1.21	P1.16-5	YYTKDTNNN**N**NLTLVP	−	III
2010550	B	4	P1.12-1	P1.16-8	YYTKD**K**NN**A**LTLVP	−	III
00151	C	Nt	P1.5-1	P1.16-38a†	YYA**K**DTNNNLTLVP	−	III

[a] Loop 1 and 4 sequence subtypes named as in the *N. meningitidis* PorA Variable Region Database (http://neisseria.org); subtype P1.16-38a has not been included in the database.
[b] The changes in sequence to the P1.16 subtype are designated in bold.
[c] Reaction with anti-P1.16 monoclonal antibody MN5C11G measured in this study, conducted as described.[6]
[d] Based on the recognition by the peptide-A conjugate and OMV-induced sera, strains were categorized in: (I) strains fully recognized by either of the sera: full cross-reactivity concerning the P1.16 epitope; (II) strains little recognized by OMV sera, but not by peptide sera; the cross-reactivity of the OMV-induced sera most likely comes from the P1.7 epitope as present in the P1.7,16 OMV; (III) strains that were not recognized by either of the sera: no cross-reactivity of the sera with the P1.16 variants.

subtype P1.7,16, were included in the experiment.[29] Peptide conjugates A and D, and OMV elicited antibodies that bound whole bacteria (Figure 2). However, only peptide A and the OMV elicited bactericidal antibodies, which activated the complement system and induced killing of the bacteria *in vitro*. In the absence of a good animal model for meningococcal disease, the serum bactericidal activity assay is considered a good predictor for the protection against *N. meningitidis* serogroup B.[30] Remarkably, the bactericidal titers induced by peptide-A conjugate were higher than the titers induced by the OMV ($p < 0.01$).

Subsequently, immunizations were performed using different doses of peptide-A conjugate, ranging from 2 μg to 50 μg, where 50 μg of conjugate is the standard dose used in immunization experiments.[6] The obtained sera were compared to the sera induced by 1.5 μg of OMV, a 1/10 human dose that is generally used in mice experiments. These experiments revealed no dose-dependency ($p = 0.37$) and showed that all doses of peptide conjugate elicited bactericidal-antibody titers that were higher than the titer induced by OMV ($p < 0.01$) (Figure 3(a)).

The antibodies induced by the lowest dose of peptide-A conjugate or OMV were tested for their cross-reactivity with other strains in whole-cell ELISA (Table 1). We observed three categories of strains based on the recognition by the peptide-induced and OMV-induced sera (Figure 3(b)). Strains of the first category, including P1.16, P1.16-33, P1.16-34 and, to a lesser extent, P1.16-2, were recognized by both sera raised by OMV and peptide A. In addition, these strains were recognized by the anti-P1.16 monoclonal antibody used in our binding assay. These data indicated full cross-reactivity of the sera with these strains. Strains of the second category, including P.16-6, P1.16-32, P1.16-35 and P1.16-29, were recognized weakly by the sera induced by OMVs. Peptide-induced sera gave little or no reaction with these strains, indicating that the sera were not cross-reactive with these P1.16 variants. This is in agreement with the observation that anti-P1.16 monoclonal antibody did not recognize strains from this category. The observed cross-reactivity of the OMV-induced sera is therefore most likely due to the other epitope (P1.7) present in the OMV; this is supported by binding of anti-P1.7 monoclonal antibody to all of these strains (data not shown). Strains of the third category, P1.16-38a†, P1.16-5, P1.16-4 and P1.16-8, were not, or hardly, recognized by either of the sera, nor by anti-P1.7 and anti-P1.16 monoclonal antibodies. The OMV-induced and peptide-induced sera are not cross-reactive with these strains, indicating that P1.7 is absent and sequence and conformation of the P1.16 variants were distinct from the original epitope. In conclusion, the sera induced by peptide-A conjugate and by OMV showed same coverage of recognition of P1.16 variants.

Implications for vaccine development

In silico evaluation by molecular dynamics simulations may select peptide designs that have the highest conformational mimicry to the cognate protein, and will thus most likely be good vaccine candidates. We used peptide cyclization to reduce the flexibility of the peptide designs and restrain their conformation towards the conformation

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† Subtype variant P1.16-38a is not registered in the PorA variable region database

observed in a peptide–Fab complex. The reduced flexibility and structural mimicry imply that the restrained peptide has higher binding affinities for bactericidal antibodies than a similar linear peptide, due to the reduced loss of entropy upon binding. Though we observed this for the functional peptide A, the binding studies could not fully discriminate between peptide designs: also the non-functional peptide B had high binding affinities for both antibodies. In contrary, free molecular dynamics simulations proved to be capable of assessing the competence of the peptide designs correctly. Contrary to previous trial-and-error work on this epitope,[5,6] this method could select a peptide as having optimal conformational properties. This peptide A was the only peptide that induced a high, cross-reactive and bactericidal antibody titer in an immunization experiment in mice.

Interestingly, even a low dose of peptide-A conjugate- induced sera with antibody titers exceeding the titer obtained when using 1/10 human dose of protein in its natural conformation (as present in the OMV). This indicated that the higher immunogenicity of the peptide was not caused by the high concentration of specific antigen, but by a different, peptide-specific mechanism, possibly due to a better accessibility of the epitope. In addition, these sera of polyclonal antibodies with enhanced bactericidal activity showed a cross-reactivity among the 12 tested strains of P1.16 variants that is identical with cross-reactivity of sera obtained by OMV immunization. These data demonstrate that good, conformational peptide vaccines may perform better than OMV-based vaccines.

The structure-based method for design and selection of peptide-vaccine candidates presented here may have a higher chance of success than empirical approaches and has the advantage that only a limited number of peptides needs to be synthesized and tested in an immunological assay. When structural data are available, it could be applied to any linear B-cell epitope of any pathogen. More specifically, the cyclization method presented here may be most suitable for mimicking surface loops of outer membrane proteins in Gram-negative bacteria, as many surface loops occur in β-turn conformations.

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Edited by I. Wilson